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Kinetics and Characterization of Interferon Production by Murine Spleen Cells Stimulated with *Legionella pneumophila* Antigens

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Formalin-killed *Legionella pneumophila* bacterial cells, as well as a purified cell wall preparation (designated F-1 antigen) containing lipopolysaccharide (LPS), stimulated production of interferons (IFNs) in mouse spleen cell cultures. *L. pneumophila* whole-cell vaccine induced an IFN that was pH 2 labile and neutralized by anti-IFN-γ indicating that IFN-γ was the dominant form present. F-1 antigen induced a mixture of IFNs, depending upon the age of the culture and cell types present. In freshly prepared whole-spleen cultures and in 2-h adherent cultures, F-1 induced predominantly IFN-α/β. In whole-spleen cultures that were allowed to age for 24 to 48 h before stimulation, F-1 was seen to induce mostly IFN-γ, with low levels of IFN-α/β present. Since only IFN-α/β was produced in T-cell-depleted populations (at 2 h or at 48 h), it is suggested that T cells are responsible for IFN-γ production in aged cultures. Additionally, heat-treated F-1, *Escherichia coli* LPS, and heat-treated *E. coli* LPS all induced similar levels of IFN-γ in whole-splenocyte or nonadherent cell cultures which were incubated 48 h before stimulation. This suggests that LPS present in F-1 is responsible for IFN-γ production and that an activated cell population is required. These results show that *L. pneumophila* antigens can induce the production of various types of IFN in mouse spleen cell cultures through several mechanisms.

Legionella pneumophila is an opportunistic facultative intracellular pathogen associated with human pulmonary infection (2). Recent studies with humans and experimental animals have been concerned with mechanisms of immunity to these organisms either mediated by serum antibody or involving cellular immune responses (9, 11). Results of studies in this and other laboratories have shown that *L. pneumophila* and antigenic components thereof can stimulate various nonspecific immune responses. For example, whole-cell vaccine, sonicate, or purified somatic antigenic derivatives, including purified lipopolysaccharide (LPS), stimulate guinea pig and murine lymphoid cells, as well as human peripheral blood leukocytes, to undergo rapid blast cell transformation in vitro (5). In addition, cell-free supernatant fluids from mouse spleen cell cultures stimulated in vitro with *L. pneumophila* antigens contain antibody helper activity as well as various cytokines, including interleukins (6). As a result of these and possibly other immunomodulatory events, *L. pneumophila* has been determined to have greater adjuvancy properties than does *Mycobacterium tuberculosis* (15).

While interferons (IFNs) traditionally have been considered to be antiviral and antiproliferative proteins, results of recent studies have demonstrated their potential for altering the immune response. IFN-γ appears particularly effective in activating macrophages (7), enhancing natural killer cell activity (1), and increasing cell surface Ia antigens (20). Because of these various immunomodulatory functions, a role for IFNs in bacterial infections has been suggested. The induction of IFN-γ may be particularly relevant in the response of the host to invasion by microorganisms, initially by activating macrophages as a nonspecific line of defense, and secondly by modulating surface antigen expression on specifically responding lymphocytes. The results of the study described here show that *L. pneumophila* vaccine and an LPS-containing somatic antigen stimulate IFN production in normal mice spleen cell cultures. Different types of IFN were induced depending upon the stimulation and the treatment of the cells.

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MATERIALS AND METHODS

Animals. Inbred BDF_1 and C3H/HeJ mice (Jackson Laboratory, Bar Harbor, Maine) were used for these studies. The animals were 8 to 10 weeks of age at the time of each experiment and were given food and water ad libitum.

Lymphoid cell preparations. Mice were killed by cervical dislocation, and their spleens were removed. Cell suspensions were prepared by disrupting individual spleens in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum (Hyclone, Logan, Utah) and antibiotics (penicillin and streptomycin) with 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.) as described previously (5). The cells were washed several times in Hanks balanced salt solution (GIBCO), centrifuged at 200 × *g* at 4°C, and used unfractionated or after separation in adherent or nonadherent cell populations by allowing whole-spleen-cell preparations to adhere to wells of plastic plates for 2, 24, 48, or 72 h at 37°C, washing the wells to remove nonadherent cells with warm medium, and adding fresh medium to a final volume of 1 ml per well. The nonadherent cells were centrifuged and resuspended with fresh medium to a final volume of 1 ml per well.

***L. pneumophila* preparations.** *L. pneumophila* whole-cell vaccine was prepared as described previously (5). Briefly, *L. pneumophila* serogroup 1, obtained originally from Roger McKinney, Centers for Disease Control, Atlanta, Ga., was cultivated on buffered charcoal yeast extract medium (GIBCO) for 28 to 36 h and harvested at the log phase of growth with sterile saline by scraping the plates with a rubber policeman. The bacteria were washed several times by centrifugation (at 10,000 × *g*) with saline and killed by incubation for 24 h with 0.5% Formalin at 10^8 bacteria per ml. Killing was verified by plating samples on buffered

TABLE 1. Induction of IFNs in murine splenocyte cultures by *L. pneumophila*

In vitro stimulator[a]	IFN activity (U/ml)[b]	
	Unheated	Heated[c]
Control	<10	<10
L. pneumophila components:		
Killed vaccine (10^8 cells/ml)	100	<10
F-1 antigen (10 μg/ml)	100	100
E. coli LPS (10 μg/ml)	100	100

[a] *L. pneumophila* components were added to splenocyte cultures at the indicated concentrations, and cultures were incubated for 24 h.
[b] Values represent mean values from triplicate determinations represented as the nearest significant figures.
[c] Stimulators were heated at 100°C for 30 min before addition to cultures.

charcoal yeast extract medium. The Formalin-killed bacteria were then washed twice by centrifugation and resuspended to a concentration of 10^9 microorganisms per ml of saline. F-1 antigen was obtained as a gift from William Johnson, University of Iowa School of Medicine, Iowa City, Iowa. The antigen was prepared by extraction of whole *L. pneumophila* and fractionated on Sephadex G-200 columns as described previously (3).

T-cell depletion. T-cell-depleted cultures were obtained by incubating 10^7 splenocytes with anti-Thy 1.2 (Cappel Laboratories, Cochranville, Pa.) for 30 min at 37°C. Cells were lysed by adding low-toxicity (rabbit) complement (Cedarlane Laboratories, Ontario, Canada) and incubating for an additional 30 min. Cells were washed three times with Hanks balanced salt solution and suspended in complete RPMI. T-cell depletion was assessed by lymphoblastogenic response to 5 μg of purified phytohemagglutinin (Burroughs-Wellcome Corp., Research Triangle Park, N.C.) per ml or 10 μg of *E. coli* LPS per ml. Cells were plated at 2×10^5 cells per well in 96-well microtiter plates and incubated with mitogen for 2 days. The blastogenic response of the cells was determined by adding [^{3}H]thymidine (0.5 μCi per culture; specific activity, 2.0 Ci/mmol; New England Nuclear Corp., Boston, Mass.), and incubating for an additional 18 h. Cultures were considered to be depleted of T cells if response to phytohemagglutinin was not above that observed in medium controls while LPS response was not depressed.

IFN induction. Mouse spleen cells, either unfractionated or after separation into adherent or nonadherent cell populations, were added at a concentration of 10^7 cells per ml to 24-well plastic plates (Costar, Cambridge, Mass.) with 1.0 ml of medium per well. To each cell preparation was then added either various concentrations of Formalin-killed *L. pneumophila* vaccine or the purified somatic antigen, F-1.

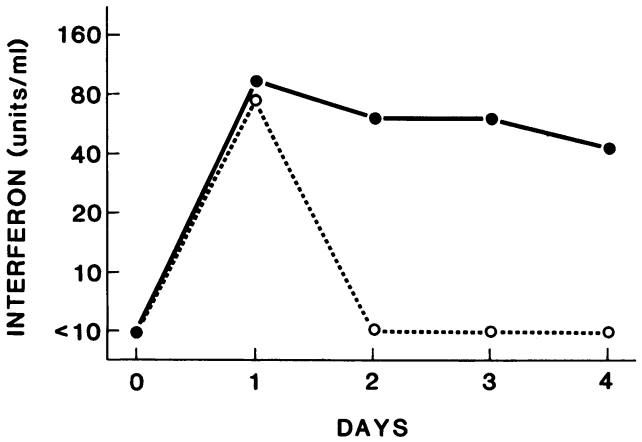

FIG. 1. Time course of IFN induction by F-1 in whole splenocyte cultures. Symbols: ●, cumulative IFN in cultural supernatants; ○, amount of IFN produced in each 24-h period after initial stimulation.

IFN assay. Serial half-log dilutions of cell-free culture supernatant fluids were made in 96-well flat-bottomed microtiter plates (Costar). To each well was then added 2×10^4 fresh L929 cells, as described previously (17). The plates were incubated at 37°C in 5% CO_2 for 24 h. Vesicular stomatitis virus, at a concentration of 4,000 PFU per well, was then added, and the plates were incubated for 18 to 24 h. One unit of IFN was calculated as the reciprocal of the dilution of the spleen cell supernatant fluid in a well which protected 50% of the cells in the monolayer from the virus-induced cytopathogenic effects. As a positive control, standard mouse IFN-α/β calibrated against reagent 6002-902-026 reference mouse IFN-α/β obtained from the National Institute of Allergy and Infectious Diseases, was used. All units are expressed in international reference units. When necessary, titers were obtained by interpolation to obtain 50% cytopathogenic effect endpoint dilutions.

IFN characterization. Cell-free culture supernatant fluids were dialyzed in 5.0-ml volumes against 500 ml of a pH 2.0 buffer (0.1 M KCl–HCl) for 24 h at 4°C to determine the acid stability of the IFN. Samples were also tested for their ability to be neutralized by rabbit anti-mouse IFN-γ, a gift from Marlyn P. Langford, University of Texas Medical Branch, Galveston, Tex., or by monoclonal anti-IFN-γ, supplied by Edward A. Havell, Trudeau Institute, Saranac Lake, N.Y. For neutralization, equal volumes of supernatant fluid and antiserum estimated to contain at least 10-fold neutralizing capacity were incubated together for 1 h at 37°C before assaying for IFN activity. Partially purified murine

TABLE 2. Characterization of IFNs induced by *L. pneumophila* vaccine

Splenocyte population	IFN (U/ml) induced after preincubation for[a]:											
	2 h			24 h			48 h			72 h		
	Control	pH 2[b]	Anti-IFN-γ[c]	Control	pH 2[b]	Anti-IFN-γ[c]	Control	pH 2[b]	Anti-IFN-γ[c]	Control	pH 2[b]	Anti-IFN-γ[c]
Whole spleen	100	<10	<10	100	30	<10	100	30	<10	170	30	<10
Adherent	30	<10	<10	30	ND[d]	ND	30	<10	<10	10	<10	<10
Nonadherent	100	30	<10	100	ND	ND	100	10	10	170	10	10

[a] Cells were allowed to adhere for 2, 24, 48, or 72 h before vaccine was added to the cultures.
[b] Supernatant fluids were dialyzed against pH 2 buffer for 24 h.
[c] Supernatant fluids were neutralized by monoclonal anti-IFN-γ before assay.
[d] ND, Not done.

TABLE 3. Characterization of IFNs induced by *L. pneumophila* somatic antigen and *E. coli* LPS

Splenocyte population (inducer added)	IFN (U/ml) induced after preincubation for[a]:											
	2 h			24 h			48 h			72 h		
	Control	pH 2[b]	Anti-IFN-γ[c]	Control	pH 2[b]	Anti-IFN-γ[c]	Control	pH 2[b]	Anti-IFN-γ[c]	Control	pH 2[b]	Anti-IFN-γ[c]
Whole spleen												
F-1	65	65	65	200	30	<10	200	100	30	200	10	<10
LPS	100	100	100	200	30	65	170	65	30	100	10	30
Adherent												
F-1	65	65	65	65	ND[d]	ND	170	100	100	100	65	65
LPS	100	100	100	100	ND	ND	100	65	65	100	65	65
Nonadherent												
F-1	65	65	65	100	ND	ND	170	65	<10	170	65	<10
LPS	100	100	65	100	ND	ND	170	30	30	170	30	30

[a] Cells were allowed to adhere for 2, 24, 48, or 72 h before F-1 or LPS was added.
[b] Supernatant fluids were dialyzed against pH 2 buffer for 24 h.
[c] Supernatant fluids were neutralized by monoclonal anti-IFN-γ before assay.
[d] ND, Not done.

IFN-γ, provided by W. Robert Fleischmann, University of Texas Medical Branch, was included in the neutralization assay as a control. Antiviral activity was characterized as being that of IFN due to lack of antiviral activity on human cells and to total prevention of development of antiviral activity on L cells treated with 3 μg of actinomycin D per ml before exposure to the supernatants. Residual antiviral activity after neutralization with anti-IFN-γ is thus referred to as IFN-α/β.

RESULTS

Normal mouse spleen cells, after incubation with *L. pneumophila* components, either whole cells or the F-1 preparation, produced IFN(s), as shown by the ability of cell-free culture fluids to interfere with the cytopathogenic effects induced by vesicular stomatitis virus on mouse L-cell cultures (Table 1). Twenty-four hours after in vitro stimulation with *L. pneumophila* antigen, an average of approximately 100 U of IFN per ml was detected in the supernatant fluids of spleen cells incubated with the whole-cell vaccine. The purified F-1 preparation also stimulated IFN formation and was essentially equivalent to that induced by the *L. pneumophila* whole-cell vaccine or, as a control, 10 μg of *E. coli* LPS per ml. The two *L. pneumophila* preparations showed differences in their ability to induce IFN activity after heating. When intact *L. pneumophila* organisms were heated at 100°C for 30 min before addition to splenocytes, no detectable IFN was induced. In contrast, heating the F-1 antigen in the same manner resulted in no significant loss of IFN-inducing activity. This was similar to the resistance to heating of the control preparation of *E. coli* LPS.

IFN activity was induced by F-1 in the cell cultures within 24 h after stimulation (Fig. 1). In these experiments, cell-free supernatant fluids were harvested at 24-h intervals from mouse splenocyte cultures stimulated with *L. pneumophila* F-1 antigen, and fresh medium without additional antigen was added to the cells. In this way, it was possible to determine the level of IFN produced during each 24-h interval after the initial stimulation. Very little, if any, IFN activity was detected in cultures which were washed and to which fresh medium was added at 24, 48, or 72 h. It is also apparent that IFN produced in the first 24-h period was stable over a 96-h period. IFN activity could be detected in the culture supernatant fluids as early as 6 h after stimulation with the *L. pneumophila* antigen and continued to increase for up to 24 h after stimulation. A similar time course was seen when the *L. pneumophila* whole-cell vaccine was used as the stimulant (data not shown).

To characterize the type of IFN produced by splenocytes stimulated with *L. pneumophila* vaccine, cells were allowed to adhere for 2, 24, 48, or 72 h, separated into adherent and nonadherent populations, and then incubated for 24 h with vaccine (Table 2). In all populations assayed, IFN-γ was predominantly produced, as shown by sensitivity to pH 2 dialysis and neutralization by anti-IFN-γ. Aging of the cultures did not have a significant effect on the level or type of IFN present. While whole-splenocyte and nonadherent populations produced higher levels of IFN, a small amount was detected in the adherent cells, possibly due to contaminating T cells or loosely adherent NK cells (8).

Incubation of splenocytes with F-1, as well as with *E. coli* LPS, stimulated different types of IFNs, depending primarily on the age of the culture (Table 3). Splenocytes adhered or incubated for 2 h produced predominantly IFN-α/β. As whole-splenocyte cultures were allowed to age, increasing amounts of IFN were detected, with the majority being IFN-γ. Similar results were seen with the nonadherent population, while the adherent cultures appeared to release more IFN-α/β, presumably due to the presence of macrophages.

Since the results of the above experiments suggested that LPS-containing *L. pneumophila* somatic antigen (F-1) induced IFN in BDF_1 mouse lymphocytes and macrophages, it was of interest to determine whether this preparation could stimulate IFN in splenocytes of LPS-nonresponder C3H/HeJ mice. The intact *L. pneumophila* vaccine stimu-

TABLE 4. Induction of IFN in C3H/HeJ splenocytes by *L. pneumophila* components

Cell population[a]	IFN activity (U/ml) induced by:		
	Vaccine[b]	F-1[c]	*E. coli* LPS[c]
Whole-spleen-cell culture	200	10	<10
Adherent cells	10	<10	<10
Nonadherent cells	100	10	<10

[a] Cells were fractionated by 2 h of adherence on plastic wells at 37°C.
[b] 10^8 killed bacteria per ml.
[c] 10 μg/ml.

TABLE 5. Effect of T-cell depletion on induction of IFNs by *L. pneumophila* components in murine splenocyte cultures

Cell population, incubation time[a]	IFN (U/ml) induced by[b]:					
	Vaccine	F-1	*E. coli* LPS	Heated vaccine[c]	Heated F-1[c]	Heated *E. coli* LPS[c]
2 h						
Whole spleen	100 (<10)	100 (100)	100 (100)	<10	100 (100)	100 (100)
T cell depleted	10 (<10)	30 (30)	30 (30)			
48 h						
Whole spleen	100 (<10)	170 (30)	170 (30)		170 (30)	200 (30)
T cell depleted	10 (<10)	30 (30)	30 (30)			

[a] Cells were incubated for 2 or 48 h before stimulators were added.
[b] Numbers in parentheses indicate units IFN after neutralization with monocolonal anti-IFN-γ.
[c] Stimulators were heated at 100°C for 30 min before addition to cultures.

lated IFN in the C3H/HeJ mouse spleen cells (Table 4). This IFN was neutralized by anti-IFN-γ, as was seen with BDF_1 mice (data not shown). In contrast, the F-1 antigen did not induce significant levels of IFN in the C3H/HeJ mouse spleen cell cultures, nor did *E. coli* LPS.

Comparison of unfractionated splenocyte and T-cell-depleted populations stimulated with *L. pneumophila* components is shown in Table 5. *L. pneumophila* vaccine induced IFN-γ in whole-splenocyte cultures, and this was dependent on the presence of T cells. F-1 and *E. coli* LPS induced IFN-α/β in both whole-spleen and T-cell-depleted cultures after 2 h of incubation. After 48 h of incubation, however, IFN-γ was primarily present in the whole-splenocyte cultures and appeared to be T cell dependent. Heating the F-1 and LPS preparations did not affect this pattern of IFN stimulation.

Since these results suggested the induction of IFN-γ by endotoxin (*L. pneumophila* or *E. coli*), whole-splenocyte cultures were stimulated by both preparations in the presence of polymyxin B (Sigma), an LPS inactivator (Table 6). As before, after a 2-h preincubation, F-1 and *E. coli* LPS induced largely IFN-α/β. In the presence of polymyxin B, however, their inductive capacity was abrogated, except for a small amount of IFN-γ induced by F-1. After 48 h, all stimulants induced IFN-γ, which was again sensitive to the presence of polymyxin B, with the exception of unheated F-1 antigen. The F-1 antigen induced significant levels of IFN-γ in the presence of the antibiotic, but this ability was lost upon heat treatment. This suggests the presence of a heat-labile component in F-1 that can stimulate the induction of IFN-γ. Induction of IFN-γ by *L. pneumophila* vaccine was not affected by polymyxin B, suggesting that a component of the whole bacterial cell which is not LPS is capable of stimulating T cells to produce IFN-γ.

DISCUSSION

The results of this study show that *L. pneumophila* vaccine and an LPS-containing somatic antigen derived from this bacterium are capable of inducing IFNs in murine splenocyte cultures. The stimulation of IFN-γ by whole bacterial cells was shown to be heat sensitive and T cell dependent, while the soluble F-1 antigen stimulated IFN-α/β in fresh culture and IFN-γ in aged splenocytes. A heat-labile IFN-γ inducing component in F-1 was also noted. It is possible that this heat-labile F-1 component is similar if not identical to the heat-labile, IFN-inducing factor in the bacterial vaccine. It is not understood why heat-treated bacterial cells do not induce detectable levels of IFN, since it would be assumed that F-1 (with a heat-stable component, presumably LPS) is still present on the cell surface. It is possible that heating of the bacterium somehow denatures outer membrane proteins such that F-1 (or LPS) is masked or rendered ineffective as an IFN inducer. The induction of IFN-γ in nonadherent spleen cell cultures by *L. pneumophila* vaccine indicates that macrophages are not required for expression of IFN activity. While IFN-γ induction normally requires an interaction between antigen-presenting cells and T lymphocytes for optimal response, this necessity is generally eliminated by the addition of 2-mercaptoethanol to the culture medium (14).

The shift from IFN-α/β stimulation to IFN-γ stimulation by F-1 (and *E. coli* LPS) is conceivably due to in vitro activation of splenocyte cultures. Since interleukin 2 (IL-2)

TABLE 6. Effect of polymyxin B on the induction of IFNs by *L. pneumophila* components

Cell population incubation[a]	IFN (U/ml) induced by [b]:				
	Vaccine	F-1		*E. coli* LPS	
		Unheated	Heated[c]	Unheated	Heated[c]
2 h					
Whole spleen	100 (<10)	100 (50)	50 (50)	65 (50)	100 (50)
Polymyxin B	100 (<10)	30 (<10)	<10	<10	<10
48 h					
Whole spleen	100 (<10)	170 (<10)	100 (<10)	100 (<10)	100 (20)
Polymyxin B	170 (<10)	100 (<10)	<10	<10	20 (<10)

[a] Cells were incubated 2 or 48 h before stimulators or polymyxin B or both was added. Polymyxin B was added at 10 μg/ml. Polymyxin B did not induce detectable levels of IFN in the absence of a stimulator.
[b] Numbers in parentheses indicate units of IFN after neutralization with monoclonal anti-IFN-γ.
[c] Stimulators were heated at 100°C for 30 min before addition to cultures.

appears to spontaneously increase in unstimulated cultures (unpublished data), an aged population of splenocytes may be more capable of responding to antigens. For example, IL-2 has been shown to induce and regulate IFN-γ production by T cells in the presence or absence of mitogens (13). This may potentiate the interaction between *L. pneumophila* components and lymphocytes. Other studies indicate that IFN-γ can activate T cells to express IL-2 receptors (12) and that both lymphokines are required for formation of cytotoxic T lymphocytes (4), suggesting that a cooperation exists between the two factors.

The apparent induction of IFN-γ by endotoxin (*L. pneumophila* or *E. coli*) may contribute to the adjuvant effect noted for LPS. Recently, a small splenic T-cell subpopulation has been reported to respond directly to LPS by proliferating (18). It is possible that the LPS may induce responsive T cells to produce a factor(s) that would amplify an ongoing response and thus become an integral part of a lymphokine cascade. An activated population then might be primed to respond to LPS by elaborating larger amounts of IFN-γ than that produced by fresh cultures. Characterization of the aged splenocyte population and potential role of IL-2 in LPS-induced IFNs is currently under investigation.

L. pneumophila has been shown to be an intracellular organism, being able to grow in monocytes and neutrophils (2). Upon activation by a cytokine-containing (IFN-γ?) preparation, monocytes were able to kill intracellular *Legionella* bacteria (10). While the presence of anti-*Legionella* antibody does not appear protective in experimental models (11), an intact cell-mediated immune response seems necessary (19). Thus, IFN may play a role in protection of the host through its modulation of cell-mediated immune functions by activating macrophages, making them more bactericidal. Additionally, it is possible that IFN-γ is part of the lymphokine cascade due to its interaction with IL-2, LPS, and potentially other cytokines. Thus, if IFN is produced in response to bacterial invasion, it could be an important determinant in the protection of the host.

It should also be noted that high fever is a consistent characteristic of legionellosis, at least in acute infection in humans. While studies from a number of laboratories have demonstrated that IFN is pyrogenic (16), it is not yet known whether in vivo induction of IFNs by *L. pneumophila* occurs, or whether IFN is responsible for this part of the pathogenicity of the organism. Further investigation of the role(s) of IFNs in bacterial pathogenesis may provide a better understanding of the mechanisms of the immune response.

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